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Enumeration of industrial *Bacillus* assemblages in commercial products with customized plate-counting assays

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ABSTRACT

The aerobic plate count assay remains among the most widespread methods for enumerating industrial *Bacillus* assemblages, as growth-independent methods are either cost prohibitive or unavailable in some areas. However, the standard plating assays used to verify the CFU count of *Bacillus*-based products are not tailored to *Bacillus* species and thus may not produce the most accurate possible estimations. Standard plating assays assume that established limits of quantification are applicable to *Bacillus* species whose colonies swarm on solid media, and that colonies of each species in a mixed-species assemblage form independently of one another on agar plates. In the present study, we examined the upper limit of quantification for an assemblage of swarming industrial *Bacillus* isolates by comparing plate count on medium with and without a swarming inhibitor with direct counts for spore suspensions of increasing endospore concentration. Additionally, we examined the impact of assemblage species composition on the evenness of colony distribution across replicate plates for four industrial *Bacillus* isolates. We compared the observed distribution of colonies across replicate plates to the expected Poisson distribution for axenic endospore suspensions, for a 3-species assemblage and a 4-species assemblage, respectively. Results suggest that customized plating assays may be more appropriate than standard protocols for the enumeration of *Bacillus*-based products, and that interactions between colonies on solid media should be considered when interpreting plating data for mixed-species *Bacillus* assemblages.

1. Introduction

The aerobic plate count (APC) assay has been a staple of benchtop microbiology for over a century (Breed and Dotterer, 1916) and continues to enjoy widespread use for the enumeration of microbially-based commercial products. Despite its popularity, the limitations of the APC assay are well-documented in the microbiological literature (Sutton, 2012; Davis, 2014). For example, dilution error and sampling error can impact the number of colony-forming units (CFUs) dispensed onto an agar plate. Furthermore, dispensing a certain number of cells onto a plate does not ensure that this number of visually distinct colonies will form. Colonies on an agar plate may arise from either a single cell or a floc of cells, and multiple colonies may merge together, impacting the number of distinct colonies apparent on the plate (Sutton, 2012). As a result, plate counting typically underestimates bacterial concentrations in tested samples (Davis, 2014). This tendency can be especially problematic when plate count assays are used to verify product label claims during the course of quality control (QC) and regulatory assessments, as a person may interpret a count within 20% of the expected CFU count as a failure to meet label claims while a trained microbiologist should recognize this result as falling within an acceptable margin of error. The counting technician is also a potential source of error in a plate counting assay. The number of colonies recorded by a skilled counting technician is not the actual number of colonies on the plate; rather, it is that technician's interpretation of colony count, and different counting technicians frequently record different counts for the same plate (Sutton, 2012). Factors such as colony morphology, colony density, and swarming behavior can all influence the technician's interpretation of colony counts. For these reasons, aerobic plate count data are best described as approximations

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Abbreviations: APC, aerobic plate count; TSA, tryptic soy agar; CFU, colony forming unit; LOD, limit of detection; LOQ, limit of quantification; TNTC, too numerous to count; TFTC, too few to count; FDA, Food and Drug Administration; ASTM, American Society for Testing Materials; BAM, Bacterial Analytical Manual; QC, quality control

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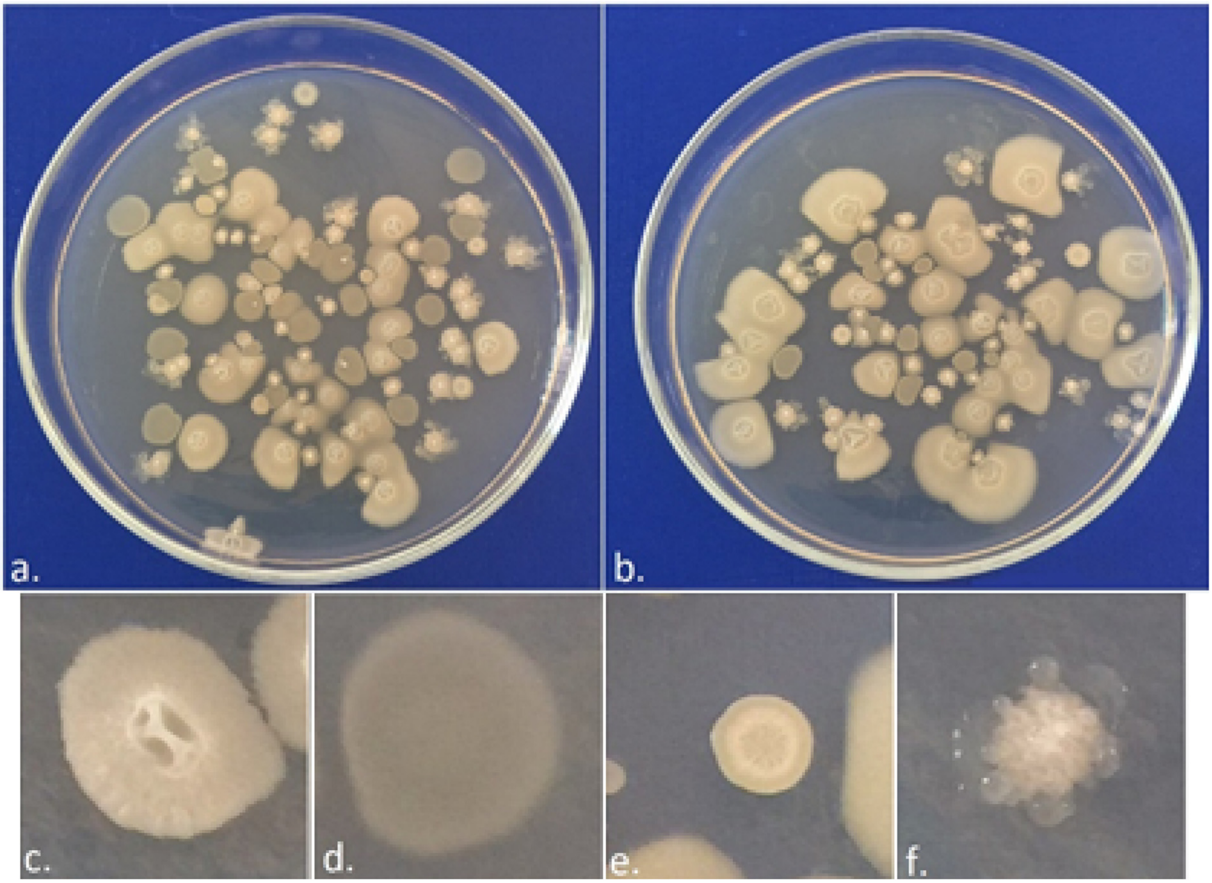

Fig. 1. Example plate counting assay shows variance between replicate plates from the same dilution bottle may be greater for some species than others. In this example from a Vietnamese laboratory, a counting technician interpreted 22 colonies of morphology (d) on plate (a), and 11 on plate (b). The same technician interpreted 7 colonies of morphology (e) on plate (a) and 2 on plate (b). Counts interpreted for morphology (c) (26 and 31) and morphology (f) (38 and 41) agreed more closely between replicates. Photo credit: Gorsuch et al. (2019).

of microbial concentration.

To produce an accurate approximation, CFU counts must be calculated from plates with colony counts that fall between an upper and lower limit of quantification (LOQ). The FDA's Bacterial Analytical Manual (BAM) (Maturin and Peeler, 2001) considers this range to be 25–250 colonies, while the American Society for Testing Materials (ASTM) defines the countable range as 20–200 colonies for a spread plate and as 30–300 colonies for a pour plate (ASTM D5465-16, 2016). However, these guidelines are not universally applicable to all microorganisms, especially organisms which swarm on solid media. Countable ranges of 25–100 colonies for soil fungi (Garrett, 1951) and of 8–80 colonies for *Aspergillus niger* (Sutton, 2012) have been suggested. This can lead to uncertainty regarding the appropriate upper LOQ for organisms for which an upper LOQ has not been empirically determined.

Due to their metabolic dormancy and resistance to environmental and chemical stressors, *Bacillus* endospores are popular inclusions in microbially-based products such as probiotics, direct-fed microbials and fertilizer amendments (Cutting, 2011). Many such products are formulated from two or more *Bacillus* species blended together in a mixed-species assemblage. Because global labeling and regulatory mandates require the accurate enumeration of *Bacillus* spores in such products, they are routinely subjected to enumeration assays. For a mixed-species assemblage, both regulators and consumers alike may demand verification of the product's total microbial concentration as well as the concentration of each species claimed on the product label. Ideally, this type of taxon-specific enumeration would be carried out with growth independent methodologies such as real-time PCR with taxon-specific primers (Gorsuch et al., 2019) or by flow cytometry with taxon-specific antibodies (Lemarchand et al., 2001). However, these methods are often unavailable or cost-prohibitive in many geographies where *Bacillus*-based products are sold. Therefore, despite its limitations and the availability of more appropriate alternatives, the APC assay continues to be the default method used by many regulators and consumers for the enumeration of products containing mixed-species *Bacillus* assemblages. When possible, qualitative enumeration of the individual species within an assemblage is accomplished in these instances by training the counting technician to recognize distinct colony morphologies, a technique which has been used in the past to distinguish colonies of different *Bacillus* species on an agar plate (Powers et al., 1976). Here again the plate counting technician becomes a source of error, as individual species counts rely heavily on the counting technician's subjective interpretation of colony morphology.

The determination of a *Bacillus*-based product's total spore count and the count of each species claimed on the label presents a number of technical challenges. As previously noted, the APC assay calculates microbial concentration from agar plates whose colony counts fall between a predetermined upper and lower LOQ; however, these limits are often different for different organisms and have not been determined for most industrial *Bacillus* species. Many *Bacillus* colonies exhibit swarming behavior on solid media, a trait which may render standard upper LOQ values (200–300 colonies) inappropriate due to the tendency of swarming colonies to merge at high densities, causing confusion among counting technicians who may misinterpret multiple merged colonies as a single colony. If quantification of individual species is required, an additional challenge presents itself: each species must be present on the plate at a level above the minimum LOQ without driving the total number of colonies per plate above the upper LOQ. Furthermore, the accuracy of microbial count estimation by plate counting depends in part upon the assumption that all colonies on the agar plate form independently of one another (Fisher et al., 1922). However, colonies of different species are known to compete for space and resources on solid media, and some colonies may inhibit the growth of neighboring colonies (Sutton, 2012). In a mixed-species *Bacillus* assemblage, these interspecies interactions may render the assumption of independent colony formation invalid and impact the consistency with which cells of less dominant species form visible colonies on replicate plates. Strong interspecies interactions can cause the less dominant species count to deviate from the expected Poisson distribution across replicate plates, negatively impacting accuracy (Fisher et al., 1922). Anecdotal reports from laboratories using plate counting assays to verify both total spore count and species-specific spore count for a mixed *Bacillus* assemblage suggest this may be the case (Fig. 1).

Until growth-independent enumeration methodologies are more universally adopted, the APC assay is likely to remain the standard by which laboratories assess both the total spore count and the relative abundance of each species within a product containing a mixed-species *Bacillus* assemblage. In the present study, we endeavored to understand the limitations of standard APC assays for enumerating these products, and to mitigate these limitations where possible. Accurate estimations of microbial concentration depend in part upon the selection of an appropriate upper LOQ for the species of interest (Sutton, 2012). We endeavored to determine the upper LOQ for an assemblage of industrial *Bacillus* isolates which produce swarming colonies on solid media. We compared the agreement of spore counts as determined via plate counting assays and direct-counting as a function of spore concentration in the plating inoculum by plating replicate samples from the same dilution bottles on solid media with and without a swarming inhibitor. Accurate estimations of microbial concentration for a mixed-species assemblage also require that colony formation for any one species is independent of colony formation by any other species. This requirement can be confirmed by showing that colony counts among replicate plates agree with an expected Poisson distribution (Fisher et al., 1922). With this in mind, we compared plate counts of *Bacillus* colonies across replicate plates with expected counts for axenic spore suspensions, for a 3-species assemblage and for a 4-species assemblage, respectively. A better understanding of issues affecting plate count accuracy may lead to the development of improved assays and more informed interpretation of plate counting data among laboratories tasked with verifying label claims for products containing assemblages of industrial *Bacillus* organisms.

2. Materials and methods

2.1. Determination of upper LOQ for swarming Bacillus colonies

An industrial *Bacillus* assemblage (*Bacillus subtilis* and *B. licheniformis*) was used for this experiment. Species identifications were confirmed via 16 s rDNA sequencing (Nelson Laboratories, Salt Lake City, UT). The product was enumerated using direct counting with a Neubauer hemocytometer and a phase-contrast microscope (Bulla et al., 1969). Spore counts were recorded for the innermost 16 squares of the hemocytometer. Direct counting was conducted in triplicate and the resultant spore counts averaged to arrive at a consensus, growth-independent count for the product of 4.5×10^9 spores per mL. Based on this value, custom dilution schemes were devised (Table 1) to target a range of 45–270 spores per plate covering the FDA BAM's suggested range of 25–250 colonies per plate. Each custom dilution scheme was executed in triplicate by completing three separate dilution experiments. Sterile peptone blank (0.01%) was used as the diluent.

Plating was carried out by spreading a 100 μL inoculum on the surface of a plate of solid media, cured benchtop for 24 h prior to use, with a freshly autoclaved glass spreader. Each dilution was plated in triplicate on two types of agar: Tryptic Soy Agar (TSA, Carolina Biological Supply, Burlington, NC) and TSA augmented with bile salts (Sigma Aldrich, Milwaukee, WI) at 0.075 g/L as a swarming inhibitor. Plates were allowed to stand for 15 min after spreading, then were inverted and held at 37 °C for 48 h (the length used in regulatory assays to allow for maximum differentiation of colony morphologies) inside a plate incubator before counting. Plates were counted by three separate technicians, with experience ranging from 1 year to 10 years, and each technician's counts were recorded separately. Counts for plates where custom (non-tenfold) dilution schemes were used were calculated using the following equation:

Table 1

Custom dilution schemes used in plating assays. The initial endospore suspension was diluted tenfold six times (10^{-6}) before being subjected to the custom dilution scheme described above, targeting a gradient of 45–270 colonies per plate. A further tenfold dilution was achieved by collecting a 0.1 mL (100 μL) sample for plating, resulting in the approximate spore concentration.

Dilution scheme	Volume of 10^{-6} dilution	Volume of sterile diluent	Expected spores/plate
90:10 dilution	10	90	45
80:20 dilution	20	80	90
70:30 dilution	30	70	135
60:40 dilution	40	60	180
50:50 dilution	50	50	225
40:60 dilution	60	40	270

$$\frac{number\ of\ colonies}{(volume\ of\ spore\ dilution)(0.1)} \times 10^8$$

2.2. Agreement of colony counts to the Poisson distribution

Axenic spore suspensions of *B. amyloliquefaciens, B. subtilis, B. pumilus* and *B. licheniformis* were obtained from an industrial fermentation company (produced by the supplier using proprietary methods that were not made available to the authors) and enumerated via direct counting as previously described. Species formed morphologically distinct colonies on TSA plates, and identities for each morphology was confirmed via 16 s rDNA sequencing (Nelson Laboratories). Direct counting values were used to prepare two different endospore suspensions: one containing comparable concentrations of *B. amyloliquefaciens* (4.5×10^8 CFU/mL), *B. subtilis* (5.7×10^8 CFU/mL), and *B. pumilus* (4.7×10^8 CFU/mL) and the second containing comparable concentrations of *B. amyloliquefaciens* (4.5×10^8 CFU/mL), *B. subtilis* (4.9×10^8 CFU/mL), *B. pumilus* (4.7×10^8 CFU/mL) and *B. licheniformis* (4.5×10^8 CFU/mL). Serial dilutions were prepared of the axenic spore suspensions, of the three-species assemblage, and of the four-species assemblage, respectively, with the targeted dilution bottle for each series having an expected concentration of $\sim 10^3$ spores per milliliter. Replicate 100 μL samples ($n = 27$ per treatment) were collected from each targeted dilution bottle with a micropipette and were spread on plates of solid media with a freshly autoclaved glass spreader. The dilution bottle was shaken manually for 30 s before each sample was collected, and samples were taken from the middle of the dilution bottle. The plating medium was Tryptic Soy agar (Carolina Biological Supply) augmented with 0.075 g/L bile salts (Sigma Aldrich) and 0.025 g/L Congo Red dye (Carolina Biological Supply), the latter added to increase visual distinction among colony morphologies. Medium was cured by allowing plates to sit benchtop for 24 h after pouring. Plates were counted by three separate technicians, with experience ranging from 1 year to 10 years, and each technician's counts were recorded separately. The colony counts interpreted in this plating exercise were recorded, and agreement with a Poisson distribution was examined using a Chi-Square Test for Goodness of Fit (McDonald, 2014) with H_0

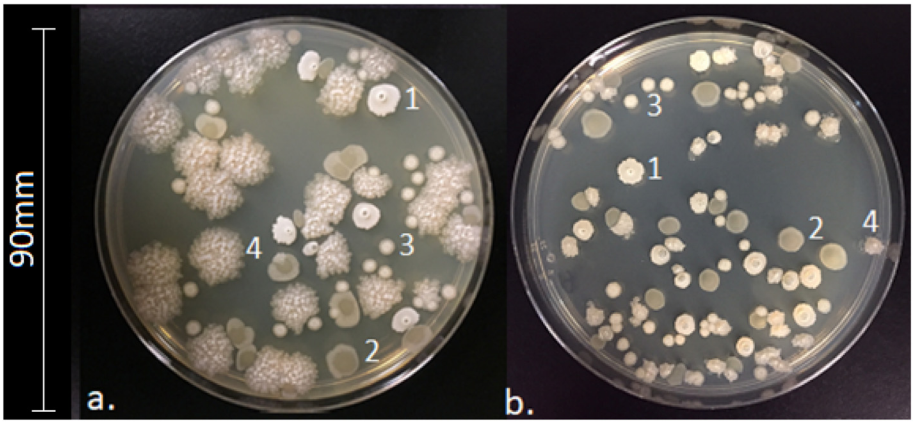

Fig. 2. Effect of bile salts as an inhibitor of swarming behavior among four industrial *Bacillus* isolates plated together in a mixed-species assemblage: *Bacillus amyloliquefaciens* (1), *B. subtilis* (2), *B. pumilus* (3), and *B. licheniformis* (4). The reduction in colony size is most clearly visible for *B. licheniformis.*

being that colony counts would be indistinguishable from direct counts at frequencies matching a Poisson distribution among replicate plates for any species in any treatment.

3. Results

3.1. Colony presentation on solid medium

The effect of bile salts as an inhibitor of swarming behavior among colonies of the four tested *Bacillus* isolates is presented in Fig. 2. Bile salts visibly reduced the size of *B. licheniformis* colonies, which on standard TSA are often observed to spread over and obscure other *Bacillus* colonies. Slight size reduction among colonies *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* was observed in the presence of bile salts, but far less dramatic.

The visually distinct colony morphologies used to differentiate the four tested species of *Bacillus* during examinations of colony distribution among replicate plates are presented in Fig. 3. Colonies of *B. subtilis* (b) and, to a lesser extent, *B. amyloliquefaciens* (a) and *B. pumilus* (c), took up Congo Red dye from the plating medium more strongly than did *B. licheniformis* (d), allowing increased visual differentiation of the four morphologies in instances where colony crowding altered colony morphology.

3.2. Determination of upper detection limits for swarming Bacillus colonies

Adding a swarming inhibitor to plating medium increased the alignment of plate counts and direct counts while decreasing inter-technician variance (Fig. 4). Plating counts which decreased below 80% of the direct count average were considered to be unsatisfactory estimations of total spore count. For plates of TSA without bile salts, values calculated from treatments with ~ 45 spores fell just inside this limit; however, plates with higher concentrations of spores produced counts which disagreed with the direct counting values. For plates of TSA with bile salts added, all treatments fell within 80% of the direct count value up to 270 spores per plate. Our data suggest that standard upper LOQ values for plate counting assays, such as the ASTM's standard of 200 colonies per spread plate, are only applicable to this swarming *Bacillus* assemblage when a swarming inhibitor is added to plating medium. Because sensitivity to swarming inhibitors is likely to vary among *Bacillus* species and their various strains, upper LOQ should be empirically determined for any *Bacillus* assemblage under consideration.

3.3. Agreement of colony counts to the poisson distribution

Significant variation from a Poisson distribution estimate of direct counts ($\alpha = 0.05$) was observed in the colony counts among replicate plates when a reportedly non-dominant *Bacillus* species, *B. subtilis*, was plated in the presence of a reportedly dominant species, *B. licheniformis*. However, this variation was not observed for *B. subtilis* in pure culture or in an assemblage with *B. licheniformis* removed (Table 2). In addition, colony counts for another reportedly non-dominant species, *B. pumilus*, did not match expected values within a Poisson distribution across replicate plates in any tested combination, including axenic cultures (Table 2). In addition, percent recovery for *B. pumilis* remained at around 78% in all conditions. In contrast, variation in colony counts on replicate plates for the nominally dominant *B. licheniformis* organism were comparable in axenic culture (p = .999) and in a four-species assemblage (p = .910). These data fit the theoretical expectations of even colony distribution and independent colony formation and compare favorably with observations suggesting that *B. licheniformis* may be the most dominant organism in the assemblage.

4. Discussion

Despite a well-known tendency to underestimate microbial activity

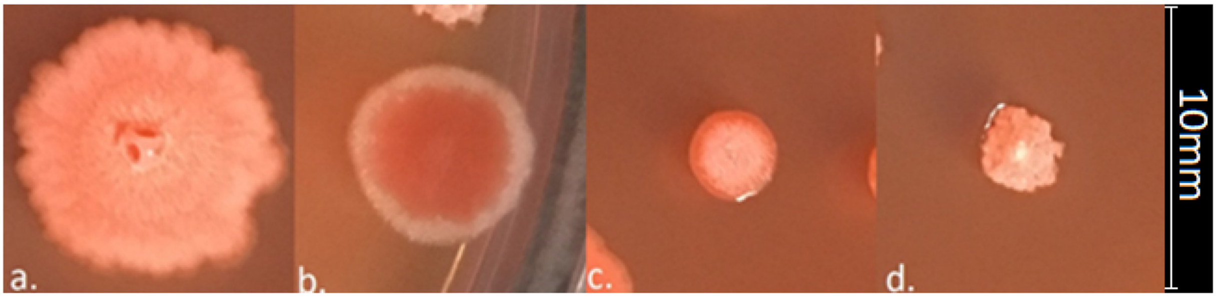

Fig. 3. Distinct colony morphologies presented by *Bacillus amyloliquefaciens* (a), *B. subtilis* (b), *B. pumilus* (c), and *B. licheniformis* (d) on solid medium. All colonies are shown to scale.

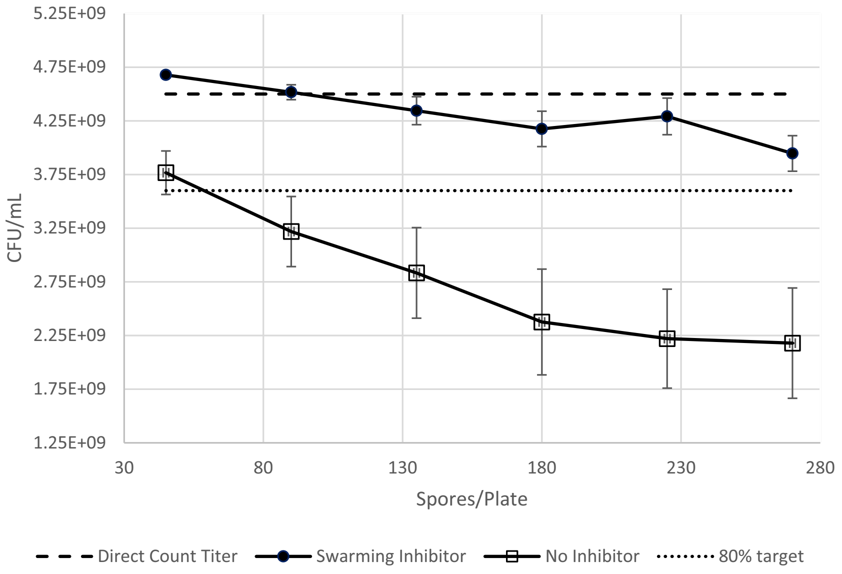

Fig. 4. Results of plating assays with and without a swarming inhibitor added to plating media. Error bars represent the standard deviation among three counting technicians above and below the data point ($n = 9$ plates per data point, three plates each from three identical plating exercises). Results calculated from inhibitor-free plates dropped below 80% of direct-counting values at counts above 45 spores per plate and inter technician variance tended to increase with spore concentration. Results calculated from plates with bile salts added as a swarming inhibitor compared favorably with direct-counting values and resulted in less variation in counts between analysts.

and the subjectivity inherent to data collection, the aerobic plate count assay remains one of the most widely accepted methods for the QC and regulatory enumeration of microbial products. Reliance on this assay for the enumeration on *Bacillus*-based products is problematic for several reasons. Limits of quantification for plate counting assays can vary depending on the organism of interest, and while customized recommendations have been issued for certain fungi no such guidelines exist for swarming *Bacillus* colonies. This may result in confusion regarding which plates should be used in the calculation of microbial concentration and, in instances where distinct colonies are difficult to interpret due to swarming behavior, in an underestimation of *Bacillus* concentration by the counting technician. The use of this method for products containing mixed-species assemblages of *Bacillus* endospores presents special challenges, especially if laboratories intend to use the method for the qualitative differentiation of species based on visually distinct colony morphologies. The accuracy of plate counting estimations depends in part upon the assumption that each species forms colonies independently of other species in the plated assemblage; however, the organisms present in a mixed-species assemblage may compete for space and resources on solid media, potentially resulting in a decreased likelihood that some species will form visible colonies and be counted by a technician.

In the present study, we examined whether serial dilutions of a microbial preparation containing 4.5×10^9 CFU/mL (via direct counting) of *Bacillus subtilis* and *B. licheniformis* produced countable plates when increasing numbers of spores (45–270, Table 1) were dispensed onto standard TSA and onto TSA augmented with bile salts as a swarming inhibitor. Countability of plates was defined as agreement within 20% of the concentration calculated through growth-independent direct counting assays. Results suggest that the upper LOQ for swarming *Bacillus* isolates on plain TSA is between 45 and 90 colonies per plate; however, with the addition of a swarming inhibitor, the upper LOQ is extended to roughly 270 colonies per plate. This LOQ compares favorably with the ASTM guideline of 20–200 colonies for a spread plate assay. We conclude that, for the tested *Bacillus* strains, the addition of 0.075 g/L bile salts to plating media inhibits swarming motility, thereby reducing colony crowding and overlap and rendering a plate that is more easily interpreted by the counting technician. This increased ease of interpretation is supported by the closer agreement of counts among analysts for plates with bile salts than for plates without depicted in Fig. 2. Therefore, in cases where at least one *Bacillus* strain within a mixed-species product exhibits swarming behavior on solid media, it may be advantageous to examine the impact of a swarming inhibitor on upper LOQ.

Agreement of colony counts to direct counts across replicate plates with an expected Poisson distribution was examined as a function of

Table 2
Fit of colony distribution for individual *Bacillus* species to expected values in identical plating aliquots from a single dilution bottle ($n = 27$ plates per treatment). Expected values were derived from microscopic counts of constituent endospore suspensions. Data in the "Observed" column is an average across all plates, the "σ" column shows standard deviations. The "p" column shows the probability of matching expected values within a Poisson distribution using the Chi squared test and statistically significant values are denoted with an asterisk. Percent recovery indicates the percentage of expected cells which produced observable colonies on agar plates.

	Expected	Observed	σ	*p* (Chi-square)	Percent recovery
B. amyloliquefaciens (axenic)	60	54	3.14	0.999	90.1
B. amyloliquefaciens (3-species)	45	44	6.38	0.59	97.8
B. amyloliquefaciens (4-species)	45	35	6.71	0.153	78.1
B. subtilis (axenic)	76	69	3.26	0.999	90.3
B. subtilis (3-species)	57	51	7.9	0.204	89.8
B. subtilis (4-species)	49	27	7.11	0.004*	54.9
B. pumilus (axenic)	63	49	10.98	< 0.001*	77.5
B. pumilus (3-species)	47	37	11.92	< 0.001*	77.7
B. pumilus (4-species)	47	37	10.65	< 0.001*	78.9
B. licheniformis (axenic)	94	88	2.41	0.999	93.6
B. licheniformis (4-species)	47	43	5.31	0.91	92.1
3-Species assemblage	149	132	15.66	0.005*	88.9
4-Species assemblage	188	143	16.09	0.006*	75.8

assemblage species composition for four morphologically distinct *Bacillus* isolates (Fig. 3). Replicate samples ($n = 27$) were collected from a single dilution bottle of each treatment and plated on solid media. Plate counts for *B. subtilis* among replicate plates only failed to match the expected distribution, as determined using a Chi-square test, in the presence of *B. licheniformis*. This variance was not observed for *B. subtilis* in a three-species assemblage with *B. licheniformis* removed or in an axenic suspension of *B. subtilis*. These results suggest that the likelihood of *B. subtilis* cells forming visible colonies is not independent of assemblage species composition - thus undermining one of the key conditions favoring accurate estimation of microbial activity - and support the hypothesis that plate counting data for a less dominant *Bacillus* species is more variable when such a species is formulated alongside a more dominant organism. Counted *B. subtilis* colonies for the axenic suspension and the 3-species assemblage constituted 90.3% and 89.8% of the expected colonies for those treatments, respectively, whereas counted colonies for the 4-species assemblage constituted only 54.9% of the expected colonies for that treatment. This observation supports the cautions issued in published literature (Fisher et al., 1922) that when the expectations of independent colony development and the agreement of counts with a theoretically even distribution are not met, CFU estimations derived from mean colony counts may be unreliable. In cases where plate counting is used to assess microbial activity in commercial products, such a discrepancy could potentially impact agreement of plate-counting data with product label claims.

A different trend was observed in the distribution of *B. pumilus* colonies on replicate plates, with significant variation from expected values in all treatments. This does not support the hypothesis that a more dominant organism is responsible for the variation observed in *B. pumilus* counts on replicate plates. Rather, these data point toward an alternate explanation for deviation from expected counts which affects all plating circumstances tested, such as low spore viability or floc formation. While these data do not suggest the same underlying cause of variation for *B. pumilus* as for *B. subtilis*, both sources of error have the same effect: a significant departure from expected values according to a Poisson distribution, which has been shown to decrease the accuracy of microbial concentrations estimated by plate counting (Fisher et al., 1922).

Quality control and regulatory assays are vital safeguards for the microbial product industry, assuring that only high-quality products are brought into the marketplace for sale. Even under the best of circumstances, use of an error-prone assay such as the aerobic plate count carries the risk of denying quality products entry into the market. This risk is exacerbated by the use of plating assays which are not optimized to the type of bacteria being enumerated. In the absence of a swarming inhibitor, applying generalized guidelines (such as the 25–250 colony countable range) to industrial *Bacillus* isolates could lead to underestimation of microbial counts even by experienced technicians in cases where the guidelines are inappropriate for the species or assemblage under consideration.

While the trends observed for this particular *Bacillus* assemblage are likely not applicable to all industrial *Bacillus* assemblages, they serve to highlight the risk inherent in treating standard plate-counting protocols as universally applicable to all microbial products. As shown here, assumptions regarding upper LOQ may not be appropriate for industrial *Bacillus* strains unless specialized plating media are used. Therefore, the development of customized plating media paired with experimental determination of upper LOQ may increase the accuracy of plate count assays for the enumeration of *Bacillus*-based products. Furthermore, some species within the *Bacillus* assemblage considered here did not present colony counts which conformed to a Poisson distribution across replicate plates, which may decrease the accuracy of CFU count estimations. As noted by Sutton (2012), the APC assay already struggles to support expectations of accuracy set forth by regulators. In cases where interactions between colonies of different species introduces additional variation into plate count data, bringing CFU count estimations in line with label claims may pose even more of a challenge. Unlike the problem of LOQ, which can be mitigated by media formulation, this is a challenge more likely to be mitigated through the establishment of reasonable margins of error for plate counting data based on an experimental understanding of how species of interest are distributed across replicate plates. Therefore, we propose that the development of customized plating assays paired with informed data interpretation may be a step toward the more accurate enumeration of *Bacillus*-based products while growth-independent methods (which arguably mitigate many of the concerns raised here) gain ubiquity.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Three listed authors (J. Gorsuch, D. LeSaint and Z. Jones) are employees of BiOWiSH Technologies, which sells products consisting of mixed-species *Bacillus* assemblages.

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